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TECHNICAL MANUSCRIPT 433

DENSITY AND BIOLOGICAL HETEROGENEITY IN EASTERN EQUINE ENCEPHALITIS VIRUS

Halvor G. Aaslestad

Edwin J. Hoffman

MARCH 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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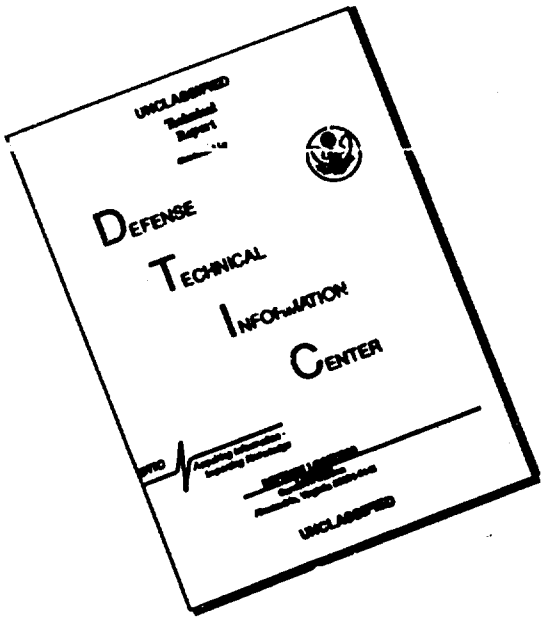

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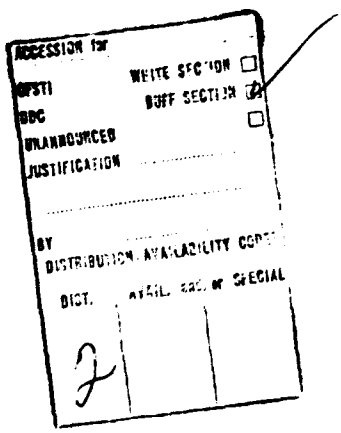

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DENSITY AND BIOLOGICAL HETEROGENEITY IN EASTERN EQUINE ENCEPHALITIS VIRUS

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Project 1C014501B71A

March 1968

ABSTRACT

Experiments in which partially purified eastern equine encephalitis (EEE) virus was centrifuged to equilibrium in CsCl revealed three bands. These consisted of a hemagglutinating particle, $\rho = 1.18$; a major infectious band, presumed to be the complete virion, $\rho = 1.20$; and a minor infectious band, $\rho = 1.22$ to 1.23.

Analysis of radioactive profiles of CsCl-fractionated EEE labeled with either $P^{32}O_4$ or uridine-H^3 indicated that most of the hemagglutinin was stripped from the complete virion. The viral origin of the hemagglutinin was verified by inhibition with specific antiserum.

Attempts to differentiate between EEE $\rho = 1.22$ to 1.23 and the complete virion ($\rho = 1.20$) showed that the denser particle was neither a viral contaminant nor a density mutant. No evidence for its being an immature form of the virus was obtained.

Results from a comparison of the kinetics of neutralization of EEE $\rho = 1.20$, $\rho = 1.23$, or unfractionated EEE with antiserum showed that the three virus populations were indistinguishable antigenically. Furthermore, all three preparations sedimented at about 250S in sucrose gradients.

These results and those obtained from CsCl rebanding experiments suggest that the denser infectious species results from a CsCl-induced alteration or breakdown of the virion and that it arises as the hemagglutinin is stripped from the surface of the complete virion.

DENSITY AND BIOLOGICAL HETEROGENEITY IN EASTERN EQUINE ENCEPHALITIS VIRUS

Many investigators have shown that considerable heterogeneity in density exists in purified virus preparations. This is easily understood if the particles are deficient or devoid of nucleic acid or are soluble antigens separating from the virus particle. However, significantly different densities also have been observed within infectious virus species, particularly among some of the enveloped viruses such as the arboviruses. Fractionations of Sindbis virus by Mussgay and co-workers[1,2] and vesicular stomatitis virus by others have demonstrated more than one infectious peak in density gradients. In our laboratory, we have recently reported[3] the presence of three virus-specific bands after equilibrium centrifugation of eastern equine encephalitis (EEE) virus in CsCl. The present report describes our efforts to characterize these bands.

The virus used in these experiments was propagated on chick embryo cell monolayers and purified from the supernatant tissue culture fluid. Purification consisted of adsorption of the virus onto $AlPO_4$ gel, followed by elution and concentration of the eluate by high-speed sedimentation. The pelleted virus was resuspended in borate-saline buffer, pH 9.0, containing 0.1% bovine serum albumin (BSA), and the suspension was adjusted to a mean buoyant density of 1.21 by the addition of CsCl. Centrifugation was at 36,000 rpm for 24 hours in a swinging-bucket rotor. The gradient was fractionated by collecting drops from the bottom of the tube. Analysis for infectivity was by plaque formation; viral hemagglutinin was detected by the method of Clarke and Casals;[4] density was calculated from the refractive index of individual fractions; and radioactivity profiles represent material insoluble in cold trichloroacetic acid (TCA) trapped on membrane filters.

A typical experiment is summarized in Figure 1. The band uppermost in the tube was diffuse and contained the majority of the hemagglutinating material. The buoyant density of the hemagglutinin band was 1.18. The mid-band contained 80 to 90% of the infectivity and is presumed to represent the complete EEE virion. Its buoyant density was 1.20. The lowermost band was composed of two distinguishable but poorly resolved subbands. This zone will be considered one band and is here represented as a saddle-shaped peak of infectivity. This material was in density range 1.215 to 1.225. It was also observed that considerable hemagglutinating activity was spread through the higher density regions of the gradient.

Figure 2 shows a gradient of $P^{32}O_4$-labeled EEE virus. The infectivity profile shown here is essentially identical to that in Figure 1. $P^{32}O_4$ peaks corresponded with the infectious virus peaks and the band of hemagglutinating material.

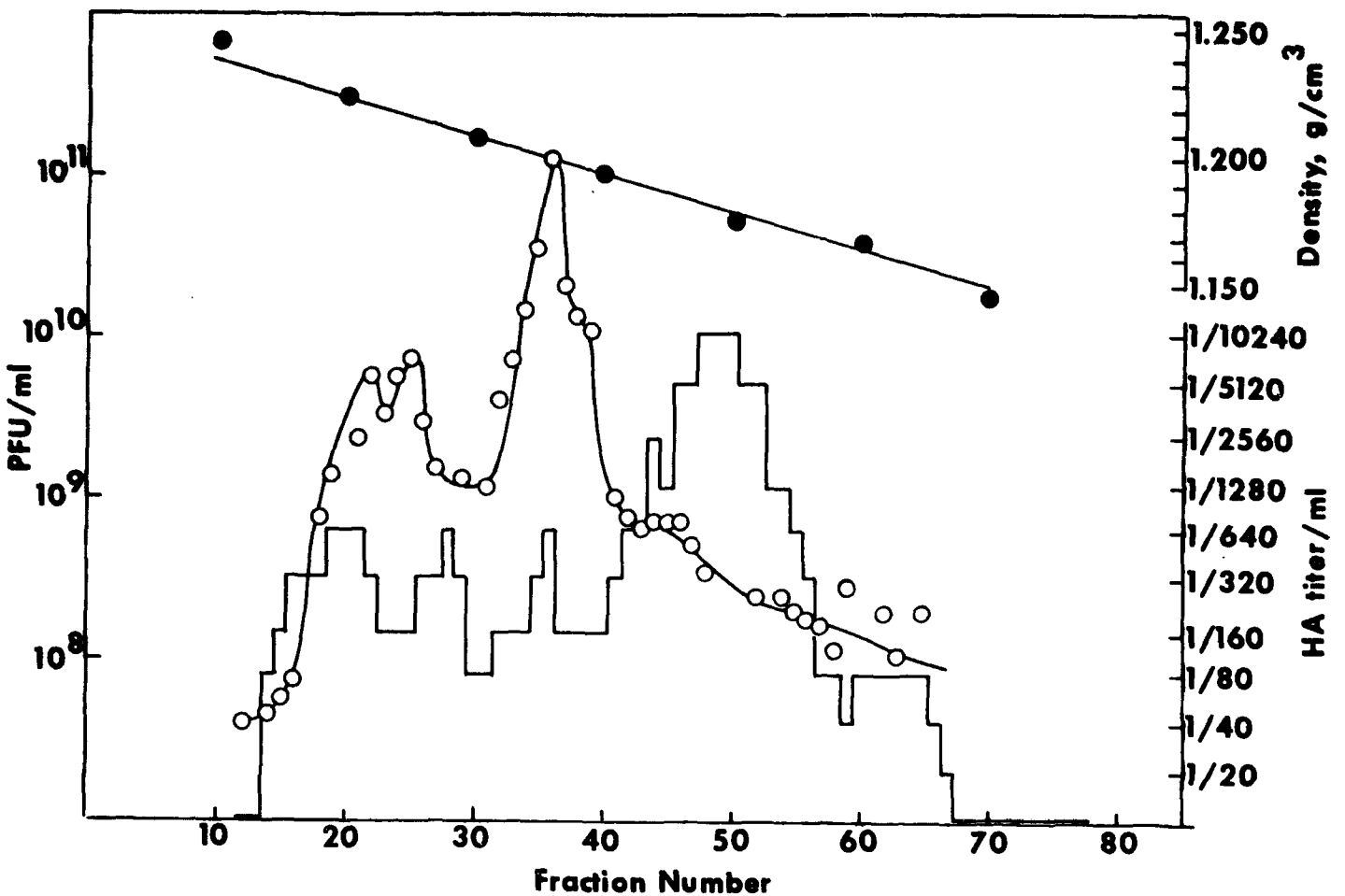

Figure 1. CsCl Density Gradient Centrifugation of Partially Purified EEE Virus. Virus infectivity (o), CsCl density (•), hemagglutinin titer (bar diagram).

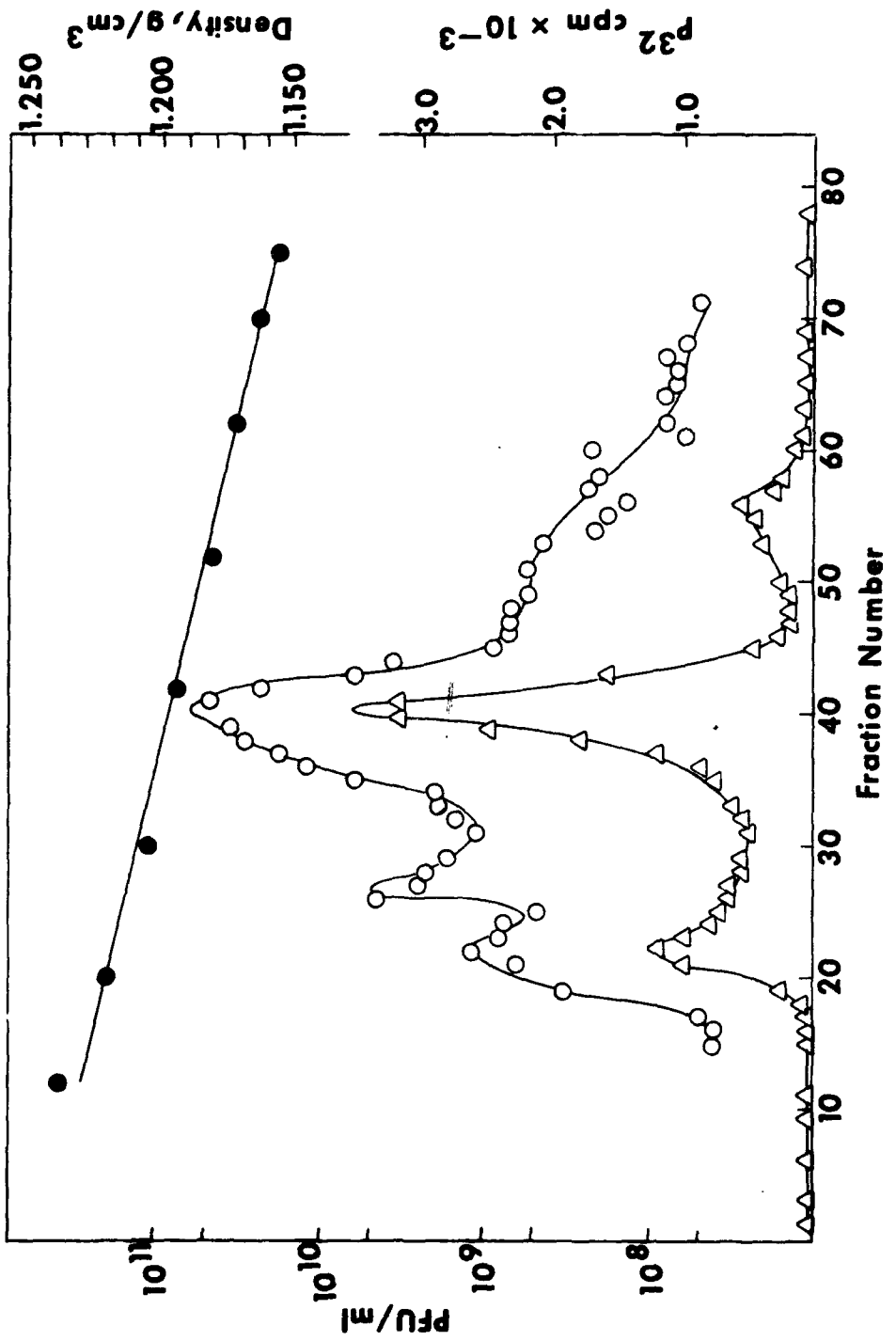

Figure 2. CsCl Density Gradient Centrifugation of Partially Purified EEE Virus Labeled with $P^{32}O_4$. Virus infectivity (o), radioactivity (Δ), CsCl density (●).

Figure 3 shows a similar experiment in which tritium-labeled uridine was used to label the virus. It is apparent that the hemagglutinating particle (ρ = 1.18) does not carry the tritium label, whereas the two regions of infectivity (ρ = 1.20 and 1.22) correspond to the labeled uridine. These density gradients indicated to us that the viral hemagglutinin has been separated from the complete EEE virion. The hemagglutinating material is that banding at density 1.18 and was most likely fragments of viral envelope and/or "spikes" or "fringe" stripped from the surface of the virion. This explanation is consistant with the label profile observed: the absence of the uridine label indicative of RNA and the presence of $P^{32}O_4$, presumably contained in phospholipid of the viral envelope.

A hemagglutinin inhibition test was performed to verify the virus-specific nature of the hemagglutinin. The results shown in Table 1 compare the hemagglutinin separated in the CsCl gradient with a standard sucrose-acetone antigen. The virus-specific nature of the hemagglutinating particle was also supported by the observation that an acidic pH was required for optimal hemagglutinin titer.

The detection of a hemagglutinating particle separate from infectious EEE virus in CsCl gradients was anticipated; however, the nature of the infectious particle banding at a density of 1.22 to 1.23 was not clear. Data from a previous report[3] from our laboratory showed that: (i) this denser form was not a virus contaminant; (ii) no proof could be found for its being a density mutant; and (iii) it appeared to be a salt-induced modification or breakdown product of the EEE virion of density 1.20. This was supported by the fact that EEE virus of density 1.20 gave rise to material banding at 1.23 and 1.18 when it was rebanding in CsCl.

The possibility still existed that this denser particle was a natural form of EEE virus, perhaps similar to that viral form described by Wecker[8] and by Colon[9] and co-workers as immature virus. By definition, immature virus should yield infectious RNA by cold phenol extraction, it should titer higher by hypertonic than by isotonic methods of assay, and it might be susceptible to ribonuclease digestion, particularly at high concentrations of ribonuclease.

The remainder of this report is confined to information on the characterization of the two infectious EEE virus bands. Table 2 presents the results of an experiment in which virus from the two infectious CsCl bands was taken from the gradient and assayed using both isotonic (beef heart infusion) and hypertonic (1 M NaCl) diluents. Both bands behaved similarly; the denser virus did not have a higher titer by the hypertonic method as would be characteristic for an immature form. In addition, ribonuclease failed to inactivate either preparation. Table 3 compares the yields of infectious RNA extracted from virus in each band by hot and cold phenol. Virus of both buoyant densities yielded infectious RNA by the cold phenol method and, in each case, from two- to threefold more when hot phenol was

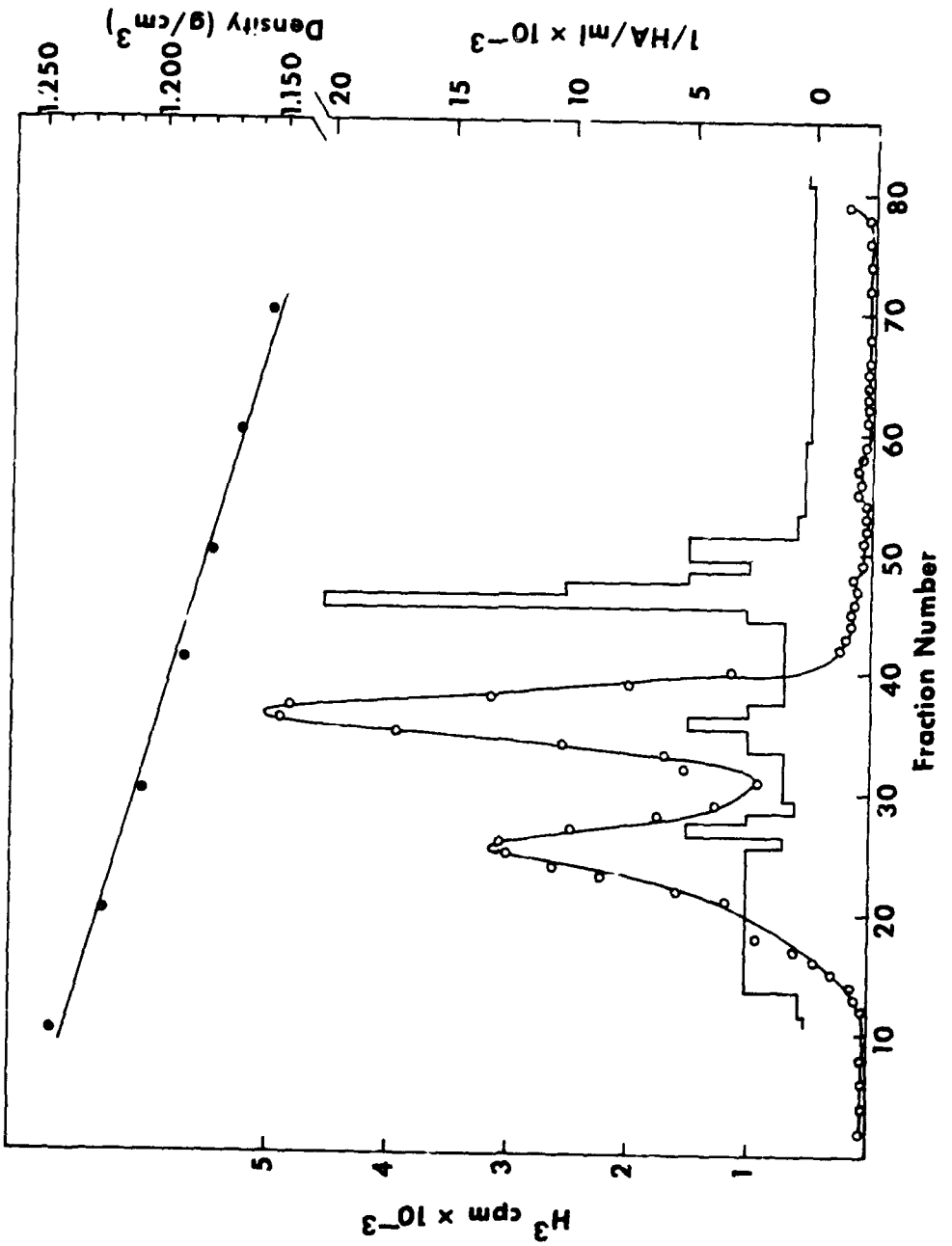

Figure 3. CsCl Density Gradient Centrifugation of Partially Purified EEE Virus Labeled with Uridine-H^3. Radioactivity (o), CsCl density (•), hemagglutinin titer (bar diagram).

employed. Because an immature form should yield infectious RNA by the cold method while the mature virion should not, this test was inconclusive. It may be that mature (intact) EEE virus (ρ = 1.20), purified to the degree this material was, became susceptible to cold phenol extraction. We feel these data offer no evidence to link EEE of density 1.23 to an immature or sub-viral form as these terms are usually understood. This is altogether consistent with experiments mentioned earlier, which indicated that EEE of density 1.20 was degraded by the CsCl to a form banding at 1.22 to 1.23.

TABLE 1. SPECIFIC INHIBITION OF THE CsCl-FRACTIONATED EEE VIRUS HEMAGGLUTININ

Test System	HA Titer
Control EEE antigen[a]	1:640
Control EEE antigen + anti-EEE serum[b]	<1:20
Control EEE antigen + normal serum[b]	1:640
EEE antigen from CsCl gradient	1:1280
EEE antigen from CsCl gradient + anti-EEE serum	<1:20
EEE antigen from CsCl gradient + normal serum	1:640

a. Control antigen was a sucrose-acetone preparation.
b. Monkey sera treated to remove nonspecific inhibitors.

TABLE 2. ISOTONIC AND HYPERTONIC INFECTIVITY AND RIBONUCLEASE SENSITIVITY OF CsCl-FRACTIONATED EEE VIRUS

Treatment	Infectivity, pfu/ml x 10^9	
	EEE ρ = 1.23	EEE ρ = 1.20
Isotonic[a]	6.5	44.0
Hypertonic[a]	0.024	0.83
Ribonuclease[b]	5.7	31.0
Ribonuclease control[b]	4.2	32.0

a. Isotonic diluent = beef heart infusion; hypertonic diluent = 1 M NaCl.
b. 20 μg ribonuclease, 30 minutes, 37 C; control was minus enzyme.

TABLE 3. INFECTIOUS RNA EXTRACTED WITH PHENOL AT HIGH AND LOW TEMPERATURES FROM CsCl-FRACTIONATED EEE VIRUS

Temperature, C	Infectivity Titer and Per Cent of Control[a]			
	EEE ρ = 1.23		EEE ρ = 1.20	
	pfu/ml x 10^5	%	pfu/ml x 10^5	%
4	1.8	0.028	4.1	0.010
56	3.2	0.049	14.0	0.032

a. EEE ρ = 1.23 titered 6.5 x 10^8 pfu/ml and EEE ρ = 1.20 titered 4.4 x 10^9 pfu/ml.

In attempts to differentiate between the two infectious bands by serologic methods, EEE virus was again fractionated to obtain virus bands of densities 1.20 and 1.23. First, a 50% plaque neutralization test was employed in which a constant amount of virus was incubated with various dilutions of antiserum. This test failed to differentiate between the two virus species. A more sensitive test was then employed based on the kinetics of neutralization (Fig. 4). In this experiment, fractionated EEE virus was diluted to about 5 x 10^6 pfu/ml and mixed with a 1:400 dilution of rabbit anti-EEE serum. The mixture was incubated at 25 C,

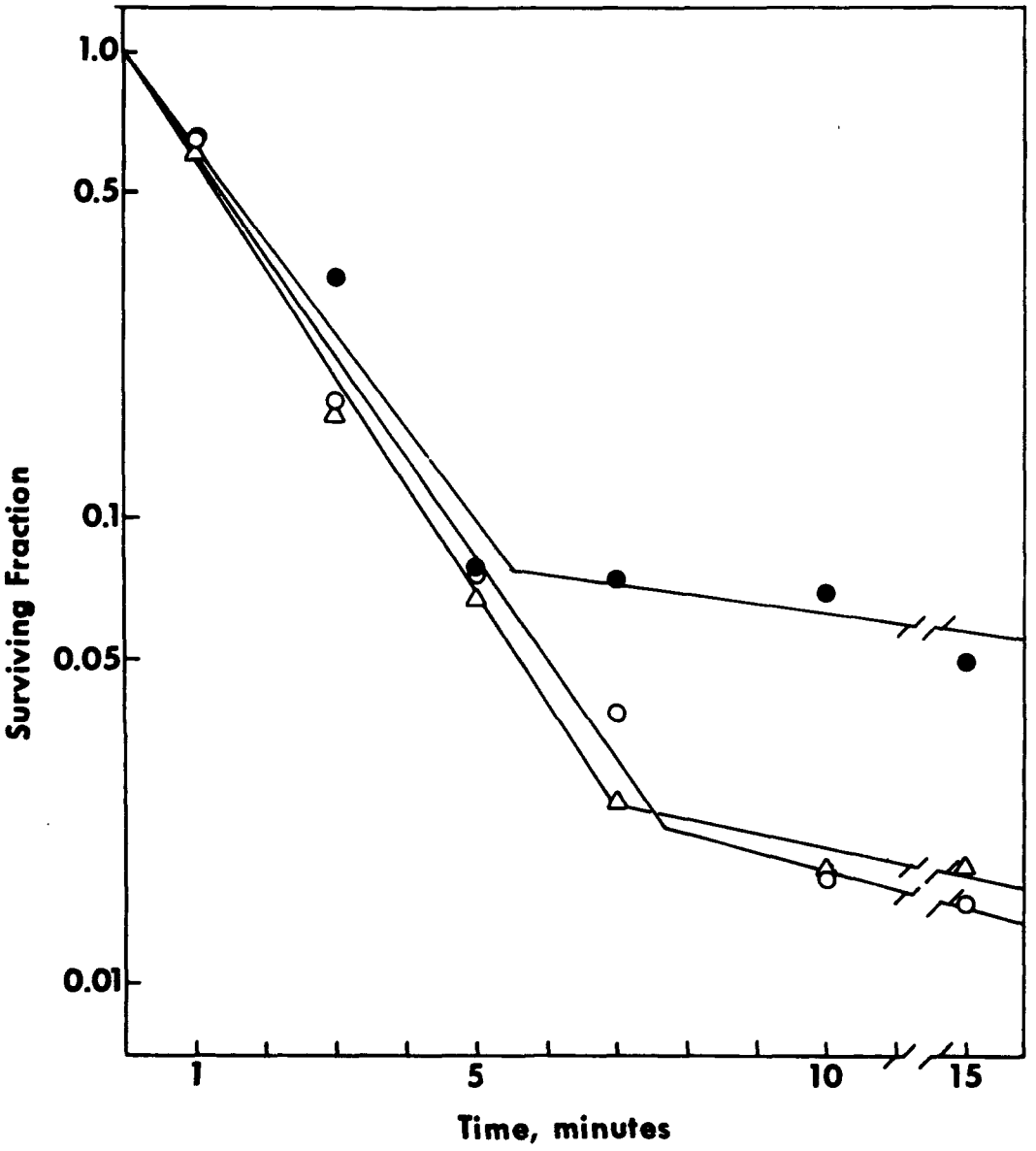

Figure 4. Antiserum Neutralization Kinetics of Fractionated and Unfractionated EEE Virus. Unfractionated EEE virus (●), ρ = 1.23 particle (○), ρ = 1.20 particle (△).

and samples were withdrawn at times shown. Samples were immediately diluted 1:100 in cold beef heart infusion and assayed for plaque formation. The EEE virus used as a control was purified both by $AlPO_4$ gel concentration and high-speed sedimentation. Controls consisting of unfractionated EEE virus as well as CsCl-banded virus incubated with normal rabbit serum maintained their titers. The slopes of the lines shown are not considered significantly different to differentiate between the fractionated virus bands.

Finally, Figure 5 shows that, when EEE virus was fractionated on CsCl, the resulting bands did not contain virus particles of different sedimentation characteristics as determined on 10 to 40% sucrose gradients. The upper third of this figure shows that unfractionated EEE virus sediments at about 250S as a single band in the sucrose gradient. When $P^{32}O_4$-labeled virus was fractionated on CsCl and the two infectious bands were isolated, dialyzed, and then centrifuged in the sucrose gradient, they could not be resolved.

In summary, we have shown that partially purified EEE virus can be fractionated into three bands during CsCl equilibrium centrifugation. The lightest band, density 1.18, is a viral hemagglutinin that may be composed of viral envelope fragments and/or "spike" material. The intact EEE virion has a buoyant density of 1.20. A slightly denser EEE infectious species banded at a density of 1.22 to 1.23. This last-named material is not a contaminant, a density mutant, or a sub-viral, immature form of the EEE virus. It cannot be differentiated from EEE virus of density 1.20 by serologic tests or by sedimentation in a sucrose gradient. It is suggested that this denser species results from a salt-induced alteration or breakdown of the complete particle. It is possible that this particle arises as the hemagglutinin is stripped from the surface of the complete virion during CsCl centrifugation.

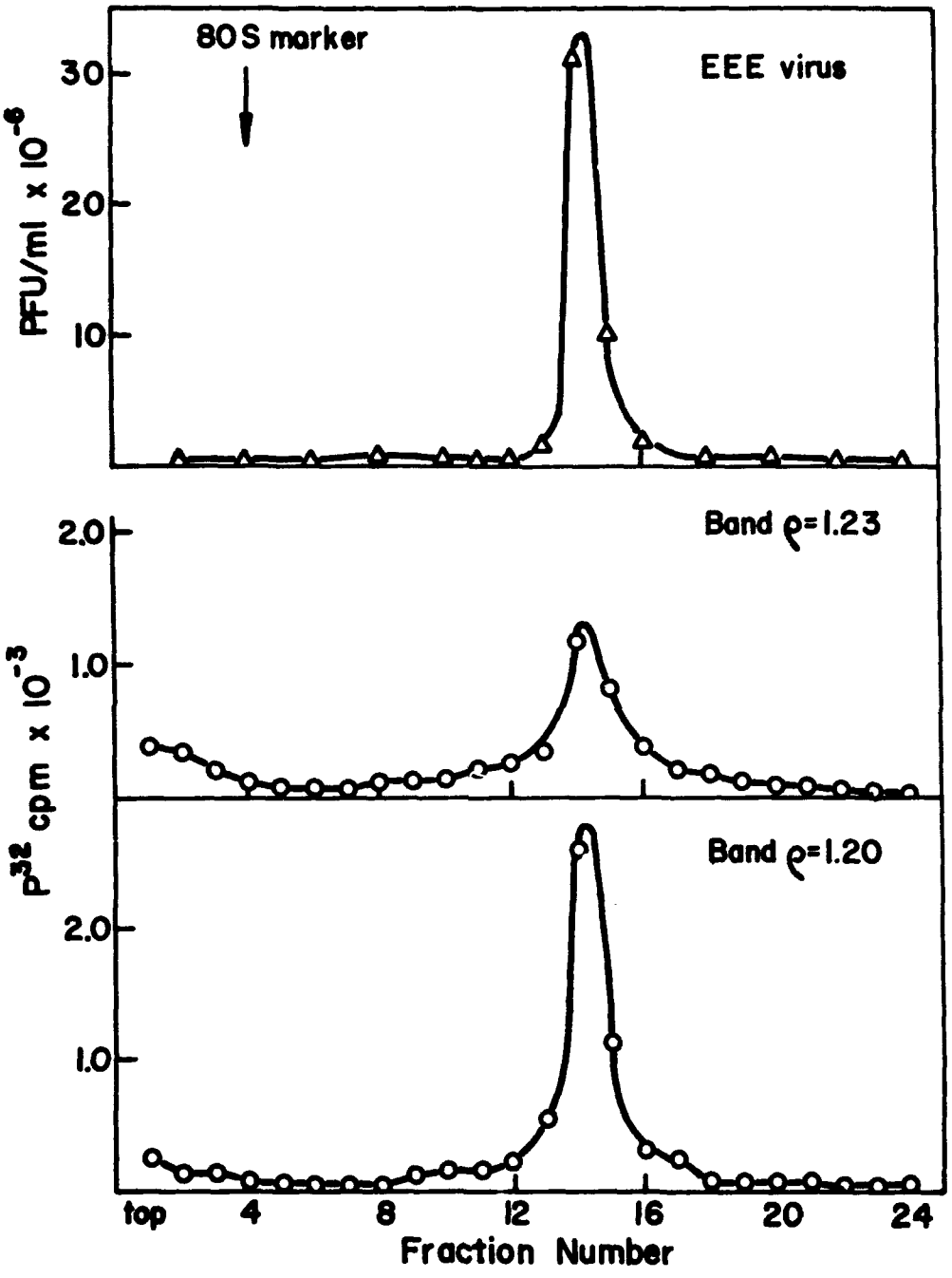

Figure 5. Sucrose Density Gradient Centrifugation of Fractionated and Unfractionated EEE Virus. Unfractionated EEE virus (top section), ρ = 1.23 particle (mid section), ρ = 1.20 particle (bottom section).

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14. Key Words

*Density	Arboviruses
*Eastern equine encephalitis	Hemagglutinin

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